STIMULATION OF SPERM FUNCTION

This invention relates to the treatment of mammalian sperm to stimulate sperm function.

Although morphologically complete and capable of vigorous motility when they leave the male reproductive tract, mammalian sperm are not immediately able to fertilize oocytes. The acquisition of the capacity to fertilize an oocyte is termed capacitation. Once capacitated, sperm can undergo the acrosome reaction, a prerequisite for penetration of the zona pellucida and fusion with the oocyte plasma membrane. A defect in this mechanism results in a condition of infertility. At present there is no real treatment for this problem.

Throughout the following description reference is made to various publications in the literature; a full reference list of these is given at the end of this specification.

Fertilization promoting peptide (FPP) has been shown to stimulate fertilizing ability in both mouse sperm (Green et al, 1994) and human sperm (Green et al, 1996a). FPP is a tripeptide (pGlu-Glu-ProNH_2) that has been detected in the prostate gland, seminal plasma and anterior pituitary of several mammals. Although no specific function has been ascribed to FPP of pituitary origin, evidence is accumulating that FPP may play an important role in regulating sperm function both in vitro and in vivo. FPP elicits biphasic responses in mammalian sperm, stimulating capacitation in uncapacitated sperm (determined by using chlortetracycline fluorescence analysis and in vitro fertilization) and then inhibiting spontaneous acrosome reactions in capacitated sperm. Sperm that have spontaneously acrosome-reacted are not able to fertilize, so both responses to FPP are important.

FPP acts by binding to its receptor (TCP11). Adenosine (also found in seminal plasma), when bound to its own receptors, can stimulate the same responses as FPP. Furthermore, using FPP plus adenosine, whether at high, stimulatory concentrations or low concentrations at which neither compound is stimulatory, stimulated capacitation more than either used singly. This suggested that FPP and adenosine act on the same signal transduction pathway (Green et al, 1996b). More recent investigations have revealed that both have a common component in the pathway, namely adenosine

receptors; the FPP receptor, TCP11, appears to interact with adenosine receptors in order to elicit a response. The responses appear to be mediated via G proteins and the adenylyl cyclase/cAMP signal transduction pathway. Stimulatory responses in uncapacitated cells involve stimulatory adenosine receptors and G proteins containing $G\alpha_s$ subunits, resulting in increased cAMP production. Inhibitory responses in capacitated cells involve inhibitory adenosine receptors and G proteins with $G\alpha_{i/o}$ subunits, resulting in reduced cAMP production (Fraser & Adeoya-Osiguwa, 1999). Agonists specific for stimulatory adenosine receptors can mimic the effects of FPP and adenosine in uncapacitated cells, while agonists specific for inhibitory adenosine receptors mimic the effects of FPP and adenosine in capacitated cells. In spite of these properties, neither FPP nor adenosine has been utilised to solve the problem with which the present invention is concerned.

Angiotensin II is an 8 amino acid hormone whose chief function is the regulation of cardiovascular and electrolyte homeostasis. The addition of angiotensin II to human sperm suspensions has been observed to stimulate sperm motility, suggesting that sperm have angiotensin II receptors and that angiotensin II may play a role in regulating sperm function (Vinson et al, 1997). This development is the subject of International patent application WO 95/32725. However, the problem to be overcome is one of stimulating sperm capacitation as distinct from sperm motility.

We have now found that, as well as stimulating sperm motility, angiotensin II stimulates the capacitation of mammalian sperm. The present invention therefore comprises the use of angiotensin II for this purpose.

The term 'angiotensin II' as used herein includes precursors and derivatives which have similar activities in vivo and in vitro. Synthetic equivalents may also be used. Production of angiotensin II involves firstly conversion of angiotensinogen to angiotensin I by renin and then conversion of angiotensin I to angiotensin II by angiotensin converting enzyme. This renin-angiotensin system is usually thought of in terms of the systemic blood-borne system, but studies during the past 2 decades have revealed the presence of elements of this system in the reproductive tract. Prorenin, the precursor of renin, and angiotensin II have both been identified in human seminal plasma.

Calcitonin is a 32-amino acid hypocalcemic hormone whose chief function is the regulation of Ca^{2+} fluxes and metabolism. There are three main phylogenetic classes of calcitonin, teleost/avian, artiodactyl and rat/human (Pozvek et al, 1997). Teleost/avian calcitonin is the most potent, with salmon calcitonin being widely used to treat human metabolic bone disorders. The term 'calcitonin' as used herein refers to calcitonin of any species including calcitonin of salmon, eel, chicken, porcine, bovine, rat and human origin, as well as precursors and derivatives which have similar activities in vivo and in vitro. Synthetic equivalents may also be used. In the early 1980s Calcitonin was identified in human seminal plasma, but negative conclusions were reached as to its effect on sperm. The first indication of a positive effect of calcitonin on sperm capacitation has been disclosed in our co-pending application WO 00/32224.

In accordance with the present invention, we have also found that combinations of two or more of the hormones referred to above and other substances specified hereinafter, produce an augmentation of capacitation which may be used to considerable advantage in therapy for infertility.

The present invention comprises a combined medication for stimulating the capacitation of mammalian sperm, comprising two or more agents each selected from one of the groups consisting of (1) calcitonin, (2) angiotensin II, and (3) a modulator of adenosine receptor activity.

The modulator of adenosine receptor activity may be selected from FPP, adenosine, and adenosine receptor agonists. For the purposes of this invention, the term 'FPP' used herein also includes precursors, related peptides such as thyrotrophin releasing hormone (TRH) and derivatives which have similar activities in vivo and in vitro.

The substances specified above, when used in combinations of any two or more, act upon mammalian sperm to stimulate sperm function significantly more than any one hormone used singly. This augmentation of the stimulatory effect can be obtained not only when using high concentrations of the hormones but also with low concentrations at which the hormones are non-stimulatory when used individually. The reason for this augmentation is not at present fully understood, but it does suggest that the different hormones are acting in some way on the same signal transduction pathway. Since it has not been found possible to increase the stimulatory effect of any one of these individual

agents simply by increasing the concentration above a certain level, the therapeutic benefit of such combinations is unexpected and highly advantageous for the treatment of fertility problems. Because these hormones act through separate receptors, defects in one or other of these, varying in individual patients, may be the cause of subfertility. Combination therapy therefore offers a more effective way of dealing with such problems.

The amounts of these agents which are effective to stimulate capacitation will depend on the extent to which sperm to be treated are initially deficient. This may be determined by assay as indicated in WO 00/3224. See below for further details.

The invention will now be further described with reference to the accompanying drawings of which:

Figure 1 shows the stimulatory effect of Angiotensin II at both 1 and 10 nM on mouse sperm,

Figure 2 shows the effect of calcitonin and angiotensin II used in combinations of both high and low concentrations with uncapacitated mouse sperm,

Figure 3 shows the effect of low concentrations of calcitonin and angiotensin II, used in combination with a low concentration of FPP none of which give a significant response when used individually,

Figure 4 shows the effect of high concentrations of FPP, calcitonin and angiotensin II used in combination, and

Figure 5 shows that the inhibition by FPP and calcitonin of spontaneous acrosome loss uncapacitated mouse sperm is abolished by pertussis toxin. Angiotensin II does not interfere with this inhibition when used in combination with FPP and calcitonin.

DEMONSTRATION OF A STIMULATORY EFFECT USING ANGIOTENSIN II

Sperm suspension preparation for chlortetracycline analysis

The contents of cauda epididymides (3-4 cauda per ml of medium) from mature TO male mice (Harlan Olac, Bicester, U.K.) were released into 2 ml modified Tyrode's medium (Fraser, 1993) in 30 mm sterile culture dishes and allowed to disperse for 5 min. Suspensions were then filtered through short columns of Sephadex G-25 (medium grade; Pharmacia, Uppsala, Sweden) to remove non-motile cells, aliquotted out to

different treatment groups and treated with nothing (control) or with the hormone of choice.

Human angiotensin II was used at final concentrations of 1 and 10 nM; FPP, used at a final concentration of 100 nM, served as a positive control. These short term incubations were carried out in 0.5 ml plastic microcentrifuge tubes at 37° C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. Following incubation, an aliquot of each treated suspension was analysed using CTC to determine whether any effects on capacitation and hence acquisition of fertilizing ability could be observed. Three replicate experiments were carried out (n=3).

In all experiments, a drop of each suspension was examined briefly for subjective motility evaluation. None of the experimental treatments had a deleterious effect on motility; judged subjectively, they all promoted more vigorous motility than that observed in untreated control samples.

Chlortetracycline fluorescence analysis

Chlortetracycline (CTC) fluorescence analysis was carried out as described by Green et al (1994). Assessments were made on an Olympus BX40 microscope equipped with phase contrast and BX-FLA epifluorescence optics using the wide blue-violet excitation cube (U-MWBV). The excitation beam passed through a 400-440 nm band pass filter and CTC fluorescence was observed through a DM 455 dichroic mirror. In each sample, at least 100 sperm were classified as expressing one of three patterns: F, with fluorescence over the entire head, a pattern characteristic of uncapacitated (non-fertilizing), acrosome-intact sperm; B, with a fluorescence-free band in the postacrosomal region, a pattern characteristic of capacitated (potentially fertilizing), acrosome-intact sperm; AR, with dull or absent fluorescence over the whole head, a pattern characteristic of acrosome-reacted (non-fertilizing) sperm.

Sperm preparation for in vitro fertilization

The contents of the cauda epididymides from 2 mature TO male mice were released into 2 ml modified Tyrode's medium in 30 mm sterile culture dishes and allowed to disperse for 5 min. Suspensions were then divided into 3 treatment groups, each in a 30 mm sterile dish, and treated with nothing (Control) or with hormone. Angiotensin II was used at a final concentration of 10 nM; FPP, used at 100 nM, again served as a positive control. Each suspension was overlaid with autoclaved liquid

paraffin and incubated for 40 min at 37° C in an atmosphere of 5% O_2, 5% CO_2, 90% N_2.

In vitro fertilization analysis

Mature female TO mice were induced to superovulate by intraperitoneal injections of 7.5 IU equine chorionic gonadotrophin and approximately 50 hr later with 5 IU human chorionic gonadotrophin (hCG). Approximately 15 hr post-hCG, oviducts were removed and cumulus masses containing the oocytes were released into medium covered with liquid paraffin. Preincubated sperm suspensions were diluted ~10 fold into medium of the same composition used for initial incubation (i.e., without or with hormone); 400 µl of diluted suspension were transferred to culture dishes, covered with liquid paraffin and oocytes were added. After 65 min co-incubation, oocytes were transferred to small droplets of control medium and at 75 min fixed with buffered formalin (4% formaldehyde in phosphate buffered saline).

Oocytes were stained with 0.75% aceto-orcein, mounted and assessed (Fraser, 1993). They were considered to be fertilized if they had resumed the second meiotic division and contained a decondensing sperm head.

Results

Epididymal mouse sperm treated with angiotensin II (AII) at both 1 and 10 nM capacitated at a significantly faster rate (***$P<0.01$) compared with untreated control sperm (Fig. 1). This was evidenced by a significantly higher proportion of B pattern cells (characteristic of capacitated, potentially fertilizing cells) and a corresponding significantly lower proportion of F pattern cells (characteristic of uncapacitated, non-fertilizing cells). It was also observed that hormone-treated cells exhibited more active motility than untreated controls. The in vitro fertilization experiments demonstrated that significantly more fertilized oocytes ($P<0.025$) were obtained with angiotensin II-treated sperm (96/174 = 55.2% oocytes fertilized) and FPP-treated sperm (81/142 = 57.0% oocytes fertilized) than with the untreated control sperm (25/128 = 19.5% oocytes fertilized).

DEMONSTRATION OF A STIMULATORY EFFECT USING COMBINATIONS OF HORMONES

Calcitonin, angiotensin II and FPP each stimulate capacitation to about the same extent (CTC analysis) when used singly. Once a significant response has been obtained, increasing the dose of an individual hormone does not increase the response markedly, e.g., 1 nM and 10 nM angiotensin II (Fig 1) elicited very similar responses, as did 100 nM and 250 nM FPP (Green et al, 1994). However, we have now demonstrated that when these hormones are used in various combinations of 2 or all 3, an augmented response is obtained. In some experiments a 'high' concentration of hormone was used (5 ng/ml salmon calcitonin; 1 nM angiotensin II; 100 nM FPP) and in others, a 'low', non-stimulatory concentration was used (0.5 ng/ml calcitonin; 0.1 nM angiotensin II; 12.5 nM FPP).

When the combination of 5 ng/ml calcitonin + 1 nM angiotensin II (high concentrations; HiCT+AII) was evaluated, the stimulatory response observed using CTC analysis was greater than that seen when either one was used individually (**$P<0.025$, ***$P<0.01$ compared with untreated controls; Fig. 2). Although neither 0.5 ng/ml calcitonin nor 0.1 nM angiotensin II (low concentrations; LoCT, LoAII) caused any detectable response in treated mouse sperm suspensions when used individually, the two low doses used in combination (LoCT+AII) produced a significant stimulatory response (**$P<0.025$ compared with the untreated controls and low dose treatments; Fig. 2). However, this result is not to be explained in terms of an additive effect of two active components since Fig. 2 shows that the components used individually at low concentrations gave no improvement in capacitation over the control, which received no treatment. These latter results suggest that the two hormones, although known to act via their own separate receptors, may be acting on the same pathway. This hypothesis would be consistent with both the augmented response to combined high doses and the significant response to the combined low doses which were non-stimulatory when used singly.

When these two hormones were used in combination with FPP, similar responses were observed. Using low, non-stimulatory concentrations, the combinations of both 12.5 nM FPP + 0.5 ng/ml calcitonin (LoF+CT) and 12.5.nM FPP + 0.1 nM angiotensin II (LoF+AII) produced a similar and significant stimulatory response

(**P<0.025 compared with untreated controls; Fig. 3). When 12.5 nM FPP + 0.5 ng/ml calcitonin + 0.1 nM angiotensin II was used (LoF+CT+AII), an even greater stimulation was obtained (***P<0.01 compared with untreated controls; Fig. 3); the magnitude of this response was similar to that obtained with 100 nM FPP (HiF). Finally, when high concentrations of all three hormones (100 nM FPP, 5 ng/ml calcitonin, 1 nM angiotensin II; HiFPP+CT+AII) were used simultaneously, a very marked stimulatory response was obtained, significantly higher (!=P<0.05) than that obtained with 100 nM FPP (HiFPP) and in untreated controls (***P<0.001; Fig. 4).

Because FPP has been shown to modulate the adenylyl cyclase/cAMP signal transduction pathway, these results suggested that calcitonin and angiotensin II may somehow also modulate this same pathway. Very recent data shown below confirm that calcitonin can significantly stimulate cAMP production in uncapacitated sperm.

Treatment	**pmol cAMP/10^7 cells** (mean ± SEM)
None (control)	21.96 ± 7.98
FPP (100 nM)	28.56 ± 9.25 *
Calcitonin (salmon, 5 ng/ml)	30.58 ± 10.80 *

***P<0.05; significantly higher than untreated control**

It should be reiterated that adenosine has been shown to utilise the same pathway as FPP; this involves adenosine receptors, with different receptor populations participating in responses to both FPP and adenosine in uncapacitated and capacitated sperm (Fraser & Adeoya-Osiguwa, 1999). New data shown below demonstrate that the combination of low FPP and low adenosine, neither of which is effective when used individually, significantly stimulates cAMP production, the amount of cAMP produced being very similar to that produced in response to either high FPP or high adenosine.

Treatment	pmol cAMP/10^7 cells (mean ± SEM)	
	2 min	4 min
None (control)	13.70 ± 1.12	12.74 ± 0.66
Hi FPP (100 nM)	18.87 ± 1.52 *	15.73 ± 1.71 *
Hi adenosine (10 μM)	18.70 ± 1.67 *	15.93 ± 1.34 *
Lo FPP (12.5 nM) + Lo adenosine (1 μM)	16.72 ± 1.34 *	17.34 ± 1.75 *

***P<0.05; significantly higher than in untreated control**

Thus combinations of compounds acting on the cAMP pathway result in increased production of cAMP in uncapacitated cells. Therefore one preferred combination is that of either FPP or adenosine, plus calcitonin and angiotensin II.

EFFECTS ON CAPACITATED SUSPENSIONS

FPP and adenosine have been shown to have a biphasic effect on mouse sperm, stimulating capacitation in uncapacitated sperm and then inhibiting the spontaneous acrosome reaction in capacitated sperm. It is important to know what effect, if any, calcitonin and angiotensin II have on capacitated sperm since treated sperm will remain in the presence of hormones for several hours. If, for example, these hormones stimulated spontaneous acrosome reactions in capacitated cells, then the effectiveness of the proposed treatment might be lessened since acrosome-reacted sperm are non-fertilizing. Therefore, the effects of calcitonin and angiotensin II used singly or in combination, including a combination of these hormones plus FPP, on capacitated sperm were investigated using CTC.

Sperm suspensions were prepared as described earlier for CTC analysis and incubated for 90 min to allow capacitation. Suspensions were then filtered to remove non-motile cells and a sample was stained with CTC. The remaining suspension was aliquotted out to different treatment groups and treated with nothing or hormone, singly and in combination: 100 nM FPP; 5 ng/ml salmon calcitonin; 1 nM angiotensin II; calcitonin + angiotensin II; FPP + calcitonin + angiotensin II. After a further incubation for 40 min (total of 130 min), sperm were prepared for CTC analysis. On the basis of a

preliminary experiment, which indicated that calcitonin had an effect while angiotensin II did not, two additional treatments were included: 100 nM FPP + 100 ng/ml pertussis toxin; 5 ng/ml calcitonin + 100 ng/ml pertussis toxin.

Results (Fig. 5) indicated that both FPP (F) and calcitonin (CT) significantly inhibited the spontaneous acrosome reaction (***P<0.01 compared with untreated controls at 130 min), whereas angiotensin II (AII) did not. Therefore, although the results obtained with uncapacitated sperm suggest that these three hormones act on the same pathway, these results indicate that more than one mechanism of action is probably involved. The inclusion of pertussis toxin (PT) abolished the effects of FPP (F+PT) and calcitonin (CT+PT), suggesting that responses in capacitated sperm to these two hormones involve G proteins with inhibitory $G\alpha_{i/o}$ subunits. The fact that angiotensin II did not inhibit spontaneous acrosome reactions suggests that a different mechanism, one not involving inhibitory G proteins, is involved in responses to this hormone. Using a combination of calcitonin + angiotensin II (data not shown) or FPP + calcitonin + angiotensin II (F+CT+AII; Fig. 5) resulted in significant inhibition of spontaneous acrosome loss (***P<0.01 compared with untreated controls at 130 min). Therefore, the inability of angiotensin II to elicit a biphasic response did not interfere with responses to FPP and calcitonin, each of which does elicit a biphasic response. These results suggest that an extended exposure of sperm to a combination of these hormones would have no deleterious effect, but would instead result in a high proportion of potentially fertilizing sperm.

CLINICAL APPLICATIONS

Therapeutic

As one highly preferred combination, we propose the combined use of FPP or adenosine, calcitonin and angiotensin II both in vitro and in vivo. The fact that this combination of hormones both stimulates capacitation and then inhibits spontaneous acrosome reactions should maximize the number of potentially fertilizing sperm (capacitated, acrosome-intact) in the sample. Individual men may have sperm with defects in one or more of the receptor-mediated responses controlled by these

hormones. Use of a mixture of all the hormones increases the chances that sperm will respond to at least one or two of the hormones in the mixture.

Uses in vitro in infertility clinics

Procedures used in infertility clinics to prepare human sperm samples for either in vitro fertilization or intrauterine insemination involve washing the sperm free of seminal plasma and hence of the hormones therein. A single hormone or a mixture containing all 3 hormones can be added to these prepared sperm samples prior to their use. Motile sperm should be selected by layering unwashed semen on top of discontinuous gradients of a dense material such as PureSperm prepared in a suitable culture medium such as Earle's medium (e.g., 95, 70 and 50% PureSperm in Earle's), centrifuging for 5-10 min at 600 g and resuspending the pelleted cells to a concentration of 5×10^6 sperm/ml in fresh medium containing the combined hormones.

For some men, addition of angiotensin II to the prepared sperm suspensions to give a final concentration of 0.1 to 100 nM (preferably 0.5 to 100 nM) would be recommended. For more general use, we recommend adding hormones to give final concentrations of FPP from 12.5 to 500 nM (preferably 25 to 500 nM) or adenosine from 0.5 to 100 μM, salmon calcitonin from 0.5 to 150 ng/ml (preferably 2 to 150 ng/ml) or human calcitonin from 2 to 2000 ng/ml (preferably 20 to 1000 ng/ml), and angiotensin II from 0.1 to 100nM (preferably 0.5 to 100 nM) in the prepared suspensions. We then recommend incubation of sperm suspensions in the presence of FPP/adenosine + calcitonin + angiotensin II for a minimum of 1-3 h at 37° C in an atmosphere of 5% CO_2, then mixing with oocytes if doing in vitro fertilization or insemination into the uterine cavity for intrauterine insemination.

In addition to being used for men with dysfunctional sperm, these procedures could be used routinely in infertility clinics for all sperm suspensions used for IVF. The hormones should have a positive effect on most sperm samples and, by increasing the number of capacitated, potentially fertilizing sperm in each sample, lower numbers of sperm could be used for insemination in vitro. This should reduce the incidence of polyspermic fertilization (fertilization by 2 or more sperm), a desirable goal since polyspermic embryos are abnormal and are never transferred.

Semen samples used for donor insemination must be frozen and stored in quarantine for at least six months before use. Freezing and thawing sperm can cause damage, in some cases making it more likely that sperm will undergo the spontaneous acrosome reaction; this would impair the fertilizing ability. The ability of these hormones, especially in combination, to inhibit spontaneous acrosome reactions might allow them to protect the sperm from such damage. All these hormones are found in seminal plasma and so would be present in samples being frozen. However, prior to freezing, semen is mixed with cryoprotectant which will reduce the concentration of hormones present. To protect the sperm from undergoing spontaneous acrosome reactions as a result of freezing and thawing we recommend adding hormones either to fresh semen prior to freezing or to thawed semen immediately after thawing, prior to insemination. We recommend adding hormones to give final concentrations of added FPP from 12.5 nM to 1 μM, or adenosine from 0.5 to 100 μM, salmon calcitonin from 0.5 to 400 ng/ml, or human calcitonin from 2 to 1000 ng/ml, and angiotensin II from 0.1 to 100 nM in the semen.

Uses in vivo

In order to increase the concentration of these hormones available to sperm at the time they enter the female reproductive tract, one can use creams, jellies or pessaries containing a mixture of FPP or adenosine, calcitonin and angiotensin II. To ensure that there is sufficient hormone to interact with ejaculated sperm, the concentration of hormones used should preferably be higher than that used for addition to prepared sperm in vitro. We recommend using preparations containing the following hormone concentrations: from 100 nM to 100 μM FPP or 10 μM to 10 mM adenosine, from 5 ng/ml to 5 μg /ml of salmon calcitonin or from 200 ng/ml to 5 μg/ml of human calcitonin and from 1 nM to 1 μM angiotensin II.

AGRICULTURAL APPLICATIONS

The present invention is applicable also to stimulating fertilizing ability of sperm in domestic animals. In many agriculturally important species (e.g., cattle, pigs, sheep) artificial insemination using either fresh or frozen/thawed semen samples is used to establish pregnancies. This is particularly important in controlled breeding

programmes where it is commercially advantageous for farmers to have specific genetically-determined traits introduced into their stock. Because FPP, adenosine, calcitonin and angiotensin II stimulate fertilizing ability in mouse sperm, a similar stimulatory effect on sperm from these various animals can be expected. In support of this, a recent study has demonstrated that FPP and adenosine have a biphasic effect on boar sperm, stimulating capacitation and inhibiting spontaneous acrosome loss (Funahashi et al, 2000). Treating the sperm with these hormones is intended to improve pregnancy rates, especially when frozen/thawed semen is being used.

Mammalian sperm are frequently damaged by freezing and thawing and this results in lower fertility. By improving the performance of the viable sperm, a mixture containing FPP or adenosine, calcitonin and angiotensin II added to the sperm preparation used for insemination should promote a higher pregnancy rate per estrus cycle, reducing the number of cycles required to ensure conception and hence reducing the overall cost of artificial insemination. At the same time, semen from animals with highly desirable traits can be used to inseminate more females because fewer cycles would be needed to ensure conception in any one female. We recommend the addition of hormones to semen samples, either prior to freezing, or after thawing but prior to insemination to give final concentrations of added FPP from 12.5 nM to 1 μM (preferably 50 nM to 1 μM) or adenosine from 0.5 to 100 μM, salmon calcitonin from 0.5 to 400 ng/ml (preferably 2 to 400 ng/ml) or porcine calcitonin from 2 to 1000 ng/ml (preferably 20 to 1000 ng/ml), and angiotensin II from 0.1 to 100 nM (preferably 0.5 to 100 nM).

For any mammalian application using angiotensin II alone we recommend a composition containing from 1nM to 1μM angiotensin II or a sperm preparation containing from 0.5 to 100 nM angiotensin II.

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